

Evidence for Two Domains of Growth Temperature for the Psychrotrophic Bacterium *Pseudomonas fluorescens* MF0

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The variations in the maximal specific growth rate of the psychrotrophic bacterium *Pseudomonas fluorescens* MF0 with respect to temperature were studied between 0 and 30°C (optimal for growth). The Arrhenius plot showed a drastic change in slope at the intermediate temperature of 17°C. Over the cold domain from 0 to 17°C, the temperature characteristic was twofold higher than over the suboptimal domain from 17 to 30°C. The macromolecular composition of exponentially growing cells was invariant over the entire range from 0 to 30°C. Variations of temperature and growth rate were independently investigated through chemostat experiments in order to characterize their respective effects on cell macromolecular composition and size. The effect of growth rate in this psychrotrophic strain is identical to that of all other bacteria assayed so far. In contrast, an original biphasic variation of total protein concentration was demonstrated in strain MF0 with respect to temperature, with a maximum at 17 to 20°C. Indeed, increasing the temperature in the chemostat resulted in a biphasic decrease in the net protein production rate: a very slight decrease below 17°C and a much larger decrease from 17 to 28°C. These results could signify an increase in the cellular protein degradation rate with increasing temperature, especially above 17°C.

Bacteria can be found at temperatures ranging from less than 0°C up to 100°C and above. Psychrotrophic bacteria are of special interest in temperate climates, since their average growth range is 0 to +40°C (21). Together with psychrophilic bacteria (–10 to +20°C), they are cold-adapted bacteria, and several studies have aimed to understand such an adaptation, in which many molecular mechanisms are obviously involved. However, it is mainly by their versatility that psychrotrophs differ from other classes, as illustrated by their predilection for habitats that undergo large thermal fluctuations (6, 28).

Little research has been devoted to adaptation of bacteria, especially psychrotrophic bacteria, to their entire temperature growth range. The variation in specific growth rate as a function of temperature is commonly studied by using the modified version of the Arrhenius equation [$k = A \exp(-\mu/RT)$], in which k is the specific growth rate, A is the collision or frequency factor, μ is the temperature characteristic, R is the universal gas constant, and T is the absolute temperature] (3, 17). The Arrhenius profiles of most bacteria are characterized by a linear portion in an average 20°C suboptimal range, which is called the normal or Arrhenius range. Below and above the normal range, deviations of the thermodependence of growth from the Arrhenius law express the inability of cells to maximize their growth rate (14). In *Escherichia coli* grown over its normal range, the thermodependent variations in the growth rate were shown to result from its metabolic control by RNA and peptide elongation rates, while all other related RNA synthesis parameters remained invariant. Since the rRNA and peptide chain elongation rates varied with the temperature in similar manners, these results were sufficient to explain the invariance of the macromolecular composition of exponentially growing cells with respect to temperature, at least in mesophilic bacteria within their normal range (4, 19, 29, 30). Furthermore, the fact that the steady-state levels of most cel-

lular proteins change very little throughout the normal temperature growth range led to the assumption that maximization of the growth rate is largely achieved by modulation of activities rather than by amounts of metabolic enzymes (14). However, more-recent studies performed in cold-adapted bacteria over their entire temperature growth range strongly suggest that changes in the synthesis of 10 to 15% of cellular proteins result from active regulatory mechanisms. Proteins which are synthesized preferentially or exclusively at certain temperatures (high or low) have been termed acclimation proteins (heat or cold, respectively) (1, 2, 11, 27, 34).

In the psychrotrophic bacterium *Pseudomonas fluorescens* MF0 whose optimal growth temperature is 30°C, several exported enzymatic activities (extracellular protease and lipase and periplasmic phosphatases) are maximal in cells grown at the intermediate temperature of 17°C (7, 20). Although co-regulated by the growth temperature, production of these enzymes is variously dependent on other growth conditions such as the medium composition and growth phase (5, 12). Furthermore, the kinetics of production of lipase and protease as a function of growth rate are dependent on the temperature (8). The permeability of the outer membrane to the β -lactamine mezlocillin is also modified around 17°C (22). These results could be related to a more general mechanism of temperature adaptation of this bacterium concerning the entire range of growth temperature, which prompted us to investigate the effect of this range on global physiological characteristics. We showed that in the suboptimal temperature range of the psychrotrophic bacterium *P. fluorescens*, two domains can be distinguished with regard to temperature dependence of maximal specific growth rate and net production rate of proteins in chemostat cultures. These domains are separated by a critical temperature around 17°C, which is the same as the optimal temperature for the production of enzymes mentioned above.

MATERIALS AND METHODS

Bacterial strain, growth media, and culture conditions. *P. fluorescens* MF0 was originally isolated from raw milk as A32 and kindly provided by R. C. MacKellar

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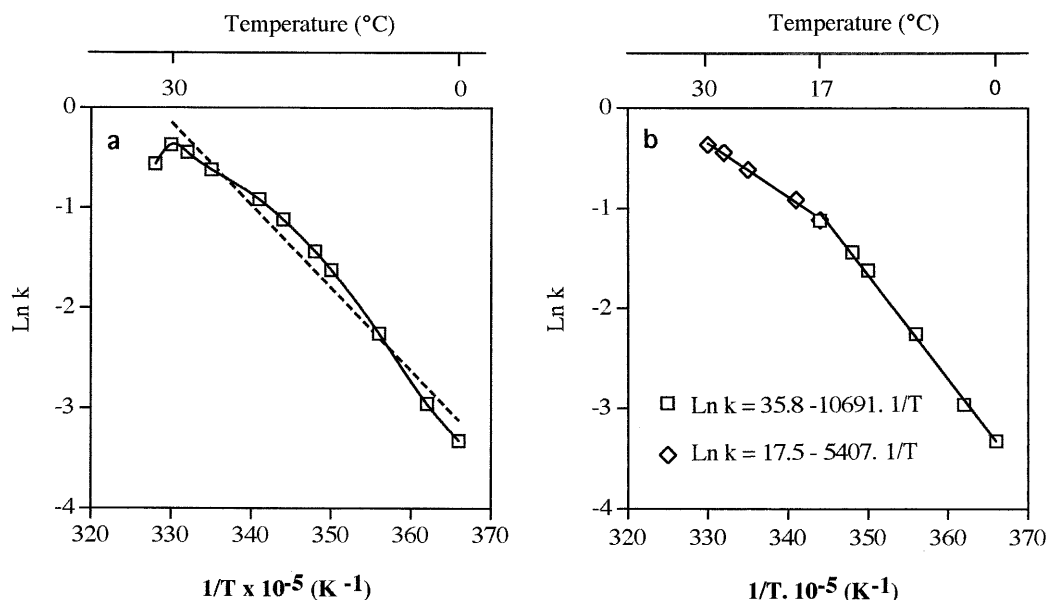


FIG. 1. Arrhenius plot ($\ln k$ versus reciprocal absolute temperature) for *P. fluorescens* MF0 in MMC. The specific growth rate k is expressed in hours^{-1} , and temperature on the x axis is expressed in Kelvin. Each datum point represents the mean of at least six independent determinations. (a) Interpolation of experimental points (solid line) and fitted curve for the Arrhenius law between 0 and 30°C ($r = 0.972$) (broken line) are shown. (b) Fitted curves for the empirical model. The lines were drawn by calculating nonlinear and linear regressions for experimental data ($r = 0.999$). Symbols: \square , 0 to 17°C; \diamond , 17 to 30°C.

(Food Research Institute, Research Branch, Agriculture Canada, Ottawa, Canada).

Liquid cultures were grown in citrate mineral salts medium (MMC) (7). This medium was supplemented, for chemostat cultivation, with the following (in milligrams per liter): $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 2.28; $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 3.00; CuSO_4 , 0.96; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.50; $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$, 0.30; H_3BO_3 , 0.30; and ferric ammonium citrate, 15.00.

A solid medium (nutrient agar [Difco] plus 10% [vol/vol] ultrahigh-temperature sterilized skim milk) was used for determination of viable cell counts.

Batch cultures were grown in Erlenmeyer flasks under rotatory agitation (180 rpm) in a water bath with a thermostat (New Brunswick) at the desired temperatures. Medium volume was 10% of the total flask volume. Cells were collected in mid-exponential phase and early stationary phase and assayed for determination of cellular proteins and RNA, and cell size was measured. Continuous cultures were carried out in MMC under carbon limitation as previously described (8). Four residence times were allowed after each modification of growth temperature or dilution rate, so that steady-state conditions were achieved. For each steady-state condition, the culture was sampled at least twice, at an interval of one residence time.

Growth was monitored by turbidity (optical density) measurements at 580 nm in a Spectronic spectrophotometer (model 601).

Maximal specific growth rate was established at each temperature in batch culture by linear regression analysis to determine the slope of the linear part of the semilogarithmic plot of optical density against time.

Determination of protein and RNA concentrations. Protein and RNA concentrations expressed in percentages of cell weight (dry weight) were determined from triplicate culture samples. Cells from 1-ml culture aliquots were precipitated at 0°C with 100- μl portions of 6 M trichloroacetic acid. The acid-insoluble material was collected by centrifugation (12,000 \times g, 10 min) and washed three times with cold distilled water (0°C). Cell pellets were resuspended in 0.2 M NaOH and kept at 20°C for about 18 h, after which 100 μl of the alkaline extract was removed for protein determination. The protein concentration was measured by the method of Lowry et al. (18), with serum albumin (fraction V; Sigma) as a standard. The remaining alkaline extract was treated at 0°C with an equal volume of 0.5 M perchloric acid and centrifuged (12,000 \times g, 10 min). The A_{260} of the supernatant was measured. Calibration with rRNA from *E. coli* W (Sigma) indicated that an A_{260} of 1 corresponds to an RNA concentration of 47.9 $\mu\text{g}/\text{ml}$.

Determination of dry weight. A culture volume (25 ml) was filtered through preweighed filters (Millipore GTTP) (pore size, 0.22 μm ; diameter, 45 mm). After the filters were washed twice with 20-ml portions of 0.1 M potassium phosphate buffer (pH 6.8), the filter pads were dried to constant weight. The correlation between the dry weight and turbidity of cultures, determined from 10 independent experiments, was found to be $0.65 \pm 0.06 \text{ mg/ml/optical density}$. This factor, which represents the buoyant cell density, was shown to be constant even when the average cell size varied twofold in chemostat experiments.

Determination of average cell mass. Cells from culture samples were counted

directly in a Thoma chamber with a phase-contrast microscope (Olympus BH2). The average cell mass was obtained by dividing the weight (dry weight) of the culture by its total cell concentration.

Determination of average cell size. Samples of culture were directly applied to agar-coated slides as described by Pfenning and Wagener (23) and observed by phase-contrast microscopy with an Olympus microscope (BH2) with 1,000-fold magnification. An Olympus camera and Pan F film were used for photography. Images were numerically acquired by Optimas software. The brightness and contrast of the entire image were adjusted to reflect the appearance of the original as closely as possible. The image of each cell is limited by a contour. The surface area of the image is measured by the number of pixels contained in the interior of the contour. Each cell surface is then assimilated to a circle of diameter d . The cell size, as measured by this diameter, did not have morphological significance and was expressed in arbitrary units. Cell size statistical analysis was performed by STATITCF software. Data were sorted, and cell size distribution analysis allowed determination of average cell size (\pm standard deviation) over a minimum of 100 cells per assay. A good correlation was found ($r = 0.98$) between average cell size and average cell mass, which were determined in parallel on six triplicate samples of chemostat cultures.

Viable cell counts and determination of plating efficiency. Culture samples were diluted in a 9-g/liter NaCl solution pre-equilibrated at the temperature of the culture. Colonies grown on nutrient agar plus 10% (vol/vol) ultrahigh-temperature-sterilized skim milk were counted after incubation of plates at the same temperature. Plating efficiency was determined as the quotient of viable cell concentration/total cell concentration.

RESULTS

Effect of temperature on the maximal specific growth rate. The logarithm of the maximal specific growth rate of *P. fluorescens* MF0 was plotted against the reciprocal of the absolute temperature to obtain its Arrhenius plot over the temperature growth range from 0 to 32°C (Fig. 1a). Above the optimal growth temperature of 30°C, the specific growth rate decreased readily, with cessation of growth occurring around 35°C in MMC (not shown). These results, together with the fact that 0°C can sustain low growth ($k = 0.036 \text{ h}^{-1}$), are consistent with the definition of a typical psychrotrophic strain (21).

When the Arrhenius law was fitted to experimental data over the range from 0 to 30°C, a low correlation coefficient was obtained ($r = 0.972$); the Arrhenius plot of *P. fluorescens* MF0

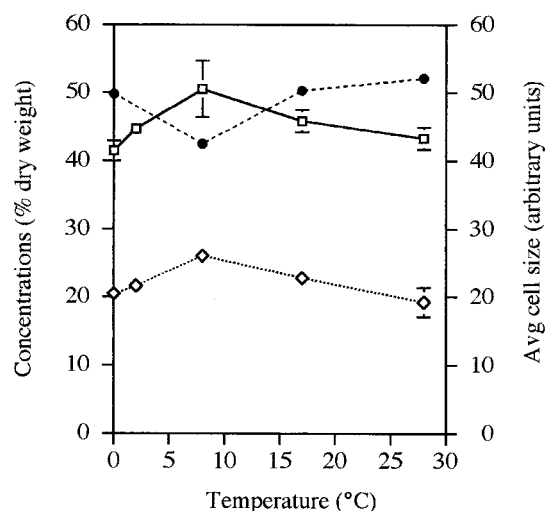


FIG. 2. Effects of growth temperature on composition and size of exponentially growing cells. Protein (\square) and RNA (\diamond) concentrations (as percentages of cell weight [dry weight]) and average cell size (\bullet) (in arbitrary units) are shown. Each datum point represents the mean of at least six independent experiments. Standard deviations are depicted by the error bars. Comparison tests of means with a significance level of 0.05 show that the protein and RNA concentration means all differ from each other and that cell size means at 0, 17, or 28°C are not different but are significantly higher than at 8°C. The average cell mass is 1.2 ± 0.2 pg.

is actually characterized by a decreasing slope between 0°C and the optimal temperature.

Nonlinear regression analysis of data over the range from 0 to 30°C and subsequent linear regressions over partial ranges led to the following empirical pattern ($r = 0.999$):

$$(i) \text{ If } \frac{1}{T} \leq 3.44 \times 10^{-3} (0^\circ\text{C} \leq T \leq 17^\circ\text{C}) \rightarrow \ln k = \alpha_1 - \alpha_2 \times \frac{1}{T}$$

with $\alpha_1 = 35.8 \pm 0.6$ and $\alpha_2 = 10,691 \pm 164$.

$$(ii) \text{ If } \frac{1}{T} \geq 3.44 \times 10^{-3} (17^\circ\text{C} \leq T \leq 30^\circ\text{C}) \rightarrow \ln k = b_1 - b_2 \times \frac{1}{T}$$

with $b_1 = 17.5 \pm 0.6$ and $b_2 = 5,407 \pm 182$.

This model thus revealed a critical point at 17°C, so that the Arrhenius profile is best fitted by two linear curves whose respective slopes determined two temperature characteristics (Fig. 1b). Between 17 and 30°C, the temperature characteristic μ of 10.7 kcal/mol (1 cal = 4.184 J) is close to those of psychrophiles (10, 15, 16). Over the cold domain from 0 to 17°C, a twofold-higher value was found ($\mu = 21.2$ kcal/mol).

Effects of temperature on composition and size of batch-cultivated cells. *P. fluorescens* MF0 was cultivated at five temperatures, 0, 2, 8, 17, and 28°C. Protein and RNA concentrations in exponentially growing cells (expressed as a percentage of weight [dry weight]) varied over this range in a similar manner (Fig. 2). Both were maximal at 8°C. This is in apparent contrast with published results showing the invariability of macromolecule concentrations in mesophilic strains over their normal range (4, 19, 30).

However, the average cell size was minimal at this same temperature of 8°C. The fact that the variation of cell size is inversely correlated with that of protein and RNA concentrations may be of significance. It might reflect the variation of another component, whose concentration would then be minimal at the temperature of 8°C.

It was therefore of interest to estimate the protein and RNA

TABLE 1. Macromolecular composition of exponentially growing cells

Growth temp (°C)	Protein content per cell ^a (arbitrary units)	RNA content per cell ^a (arbitrary units)
0	20.7	10.2
8	21.5	11.1
17	23.1	11.5
28	22.6	10.1

^a Results derived from the data of Fig. 2 by calculation of the product of protein or RNA concentrations \times average cell size for each temperature.

contents per cell for each temperature. This was done by calculation of the product of protein or RNA concentration \times average cell size. For this calculation, average cell size was considered more suitable than average cell mass, since the method used for cell size determination is more sensitive and precise. Table 1 shows that there were no significant variations in protein and RNA cell contents over the studied temperature range. According to this mode of expression of cellular composition, the behavior of this psychrotrophic strain over its entire suboptimal temperature growth range is therefore not different from that of mesophilic bacteria over their normal range.

Protein and RNA concentrations, as well as average cell size, were lower in stationary-phase cells than in exponentially growing cells, except at 0°C where they were similar (Table 2). During determination of viable cell concentrations, we observed that the cells were highly sensitive to heat and cold shocks. Even when the greatest care was taken—dilutions performed in diluent at the same temperature as that of the culture and incubation of the plate at the same temperature—the plating efficiency was rather poor for exponentially growing cells and varied with the growth temperature with a maximum at 20°C. This maximum was reached via the stationary phase, regardless of the temperature (Table 2). The significance of this deficiency in plating efficiency is not known but might reflect some lack of stress resistance in exponentially growing cells below and above 20°C.

It must be concluded that the existence of a critical point at 17°C on the Arrhenius plot had no obvious consequence on the macromolecular composition of exponentially growing cells. However, the batch cultivation method implies that changes in the growth temperature lead to concomitant changes in the growth rate. The respective effects of these parameters on cell composition and size were therefore investigated by indepen-

TABLE 2. Influence of growth temperature and growth phase on batch-cultivated cells

Temp (°C)	Stationary phase ^a			Plating efficiency ^b	
	Protein concn	RNA concn	Avg cell size	Exponential phase	Stationary phase
0	95	92	99	0.59 (0.06)	0.84 (0.06)
8	77	68	77	0.82 (0.04)	0.91 (0.02)
17	83	73	81	0.79 (0.03)	0.89 (0.08)
20	ND	ND	ND	0.95 (0.02)	0.93 (0.03)
28	90	77	86	0.67 (0.07)	0.89 (0.04)

^a Concentrations and sizes determined in stationary phase are expressed as the percentages of those in exponential phase. ND, not determined.

^b Plating efficiency was determined as the quotient of viable cell concentration/total cell concentration. The mean values from three determinations are shown; standard deviations are given in parentheses.

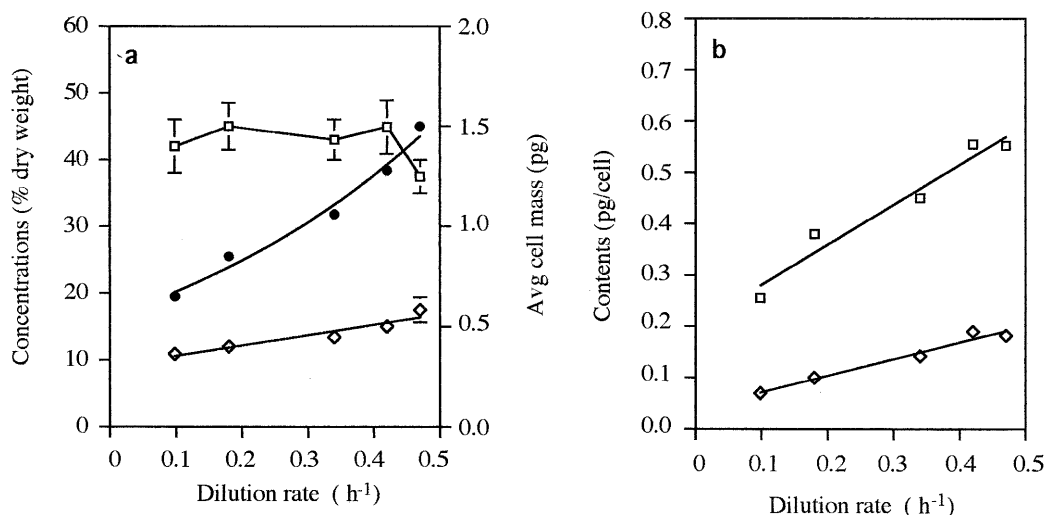


FIG. 3. Effects of dilution rate (growth rate) on cell composition and mass. Temperature was maintained at 28°C (near-optimal growth temperature), and cells were grown for 5 or 6 generations at each dilution rate in a chemostat under carbon limitation. (a) Protein (□) and RNA (◇) concentrations (as percentages of cell weight [dry weight]) and average cell mass (●) (in picograms) are shown. Each datum point represents the mean of three independent chemostat experiments. Standard deviations are depicted by the error bars. (b) Protein (□) and RNA (◇) contents (in picograms per cell) were determined from the data shown in panel a by calculation of the products of protein or RNA concentration \times average cell mass.

dently modifying the temperature and growth rate in chemostat cultures.

Effects of growth rate at 28°C on cell composition and mass.

The same parameters (protein and RNA concentrations and average cell mass) were determined for steady-state growing cells in a chemostat under carbon limitation. The temperature was maintained at 28°C (near-optimal temperature), while the dilution rate (growth rate) was varied between 0.098 and 0.47 h⁻¹.

Protein concentrations slightly decreased, whereas RNA concentrations increased linearly with the growth rate (Fig. 3a). In contrast, the average cell mass increased exponentially with the growth rate (Fig. 3a). The plating efficiency for che-

mostat-grown cells averaged 0.95 and was thus similar to that of stationary-phase cells.

Protein and RNA contents per cell, deduced from the above results, increased linearly with the growth rate (Fig. 3b).

Effects of temperature variations at a constant growth rate on cell composition and size. This experiment was performed in a chemostat under the same limitation, at a constant dilution rate ($D = 0.08$ h⁻¹). Temperature was lowered stepwise (3 to 5°C steps) from 28 to 8°C. A dilution rate of 0.08 h⁻¹ was chosen because it was low enough to prevent with certainty the washing out of the fermenter even at the lowest temperature assayed (8°C). Interestingly, the variation of the protein concentration (Fig. 4a) appeared biphasic, displaying a significant

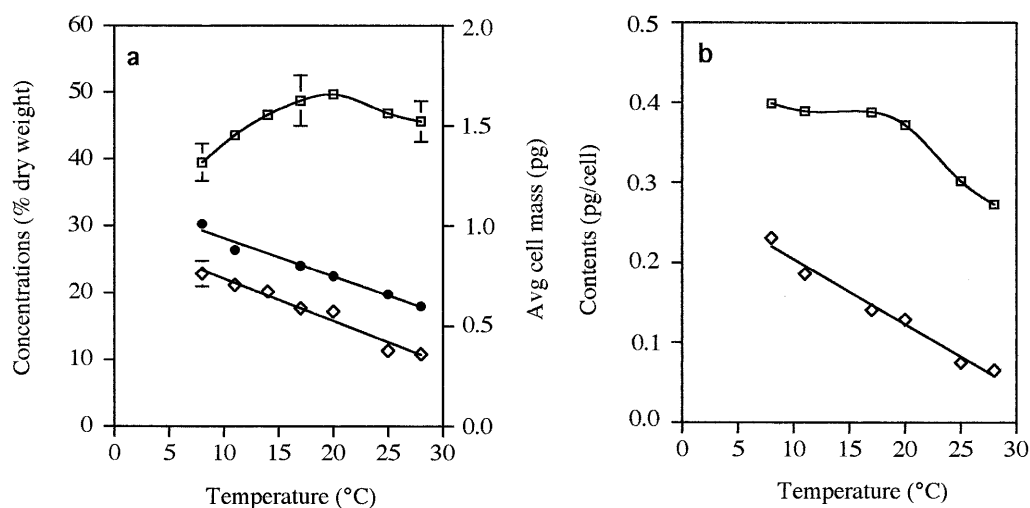


FIG. 4. Effects of growth temperature on cell composition and mass. The dilution rate (growth rate) was maintained at 0.08 h⁻¹ in a chemostat under carbon limitation, and cells were grown for 5 or 6 generations at each temperature. (a) Protein (□) and RNA (◇) concentrations (as percentages of cell weight [dry weight]) and average cell mass (●) (in picograms) are shown. Each datum point represents the mean of four independent chemostat experiments. Standard deviations are depicted by the error bars. (b) Protein (□) and RNA (◇) contents (in picograms per cell) were determined from the data shown in panel a by calculation of the products of protein or RNA concentration \times average cell mass.

maximum from 17 to 20°C. In contrast, the RNA concentration (Fig. 4a) increased markedly and linearly from 28 to 8°C, as did the average cell mass (Fig. 4a).

Protein and RNA contents per cell were therefore deduced from the above results and plotted against the growth temperature (Fig. 4b). Since chemostat cultures were grown with a constant dilution rate (growth rate) of 0.08 h⁻¹, the net production rate of proteins (or RNA) can be determined at each temperature by the product of protein (or RNA) content \times dilution rate. The cellular RNA content (like the net RNA production rate) decreased regularly when the temperature increased. In contrast, the cellular protein content, like the net protein production rate, showed a biphasic decrease: a very slight decrease over the cold domain 8 to 17°C and a much larger decrease over the suboptimal domain from 17 to 28°C (Fig. 4b).

DISCUSSION

The Arrhenius plot of *P. fluorescens* MF0 over the range from 0 to 30°C showed a drastic change in slope at the intermediate temperature of 17°C, thus defining two linear portions. There is a cold domain from 0 to 17°C in which the temperature characteristic is twofold-higher than over the suboptimal domain from 17 to 30°C. Interestingly, the latter, which is usually called the normal temperature range, is thus more restricted than those observed for most bacteria (20°C average) (15–17).

Chemostat studies showed that the effects of growth rate (dilution rate) on cell composition and mass in this psychrotrophic strain are identical to those found in all other bacteria assayed so far, both cold adapted and mesophilic (9, 31, 32). In contrast, an original biphasic variation of protein concentration was demonstrated in *P. fluorescens* MF0 with respect to temperature, with a maximum at 17 to 20°C. Such a behavior has not yet been described for other bacteria, in which temperature profiles of protein variations, although different from each other, are all monophasic. Increasing the growth temperature in the chemostat leads to an increase in protein concentration in the mesophilic *Aerobacter aerogenes* (33), while the opposite response (i.e., a decrease in protein concentration) was observed in two psychrophilic bacteria (9, 13).

Taking into account the temperature ranges assayed by these researchers, we argue that the monophasic patterns they report reflect temperature variations within the normal range of the studied bacteria, whereas *P. fluorescens* MF0 was studied over a wider range, including the critical point of its Arrhenius plot at 17°C.

It should be noted that the sum of protein and total RNA concentrations is nearly constant in *P. fluorescens* MF0 between 8 and 20°C (65.5% [dry weight]) and then decreases at 25 and 28°C (58.4 and 56.5% [dry weight], respectively). This decrease reflects a decrease in both protein and RNA concentrations from 20 to 28°C and could be due to the concomitant increase in the concentration of another component. In this case, increasing the temperature from 8 to 28°C might result in a biphasic decrease in the RNA concentration. The fact that a regular and linear decrease in RNA was observed is not consistent with the above hypothesis. Therefore, the decrease in protein concentrations which occurred from 20 to 28°C must reflect an active response to the increase in temperature.

In fact, we showed that the net production rate of proteins decreased very slightly from 8 to 17°C but decreased more readily from 17 to 28°C. This net production rate is the result of the synthesis and degradation rates at each temperature. The protein synthesis rate can be assumed to be rather invari-

ant as a result of compensating variations in the ribosome concentration (i.e., RNA) to maintain a constant growth rate in the chemostat (32, 33). The above result would then suggest that the protein degradation rate might increase with the growth temperature, especially from 17 to 28°C, i.e., throughout the suboptimal domain. If confirmed by new experiments, this would support the observations made in another psychrotrophic bacterium, *Arthrobacter globiformis*: indeed, the intracellular proteolytic activities of cells grown at low (10°C) and intermediate (20°C) temperatures were much lower than those of cells grown at 32°C (supraoptimal temperature) (24–26).

Previous studies demonstrated that 17°C was the optimal temperature for the production of several exported enzymes and the expression of at least two of the corresponding genes (5, 7, 8, 12, 20). This temperature has now been shown to have a broader significance, since it was identified as the limit between a cold domain (0 to 17°C) and a suboptimal domain (17 to 30°C). Indeed, the maximal specific growth rate and the net production rate of cellular proteins were affected differently by the growth temperature, according to the temperature domain. Furthermore, the plating efficiency for exponentially growing cells is maximal at the narrow temperature of 20°C. It may be postulated that growth temperature regulates several metabolic pathways in different and independent ways but that there are few “thermometers” that all sense a temperature change around 17°C. Moreover, the comparison of temperature effects on the protein metabolism of mesophilic, psychrophilic, and psychrotrophic bacteria has revealed differences that had not been previously observed. This raises the question of a possible general significance of our results with regard to psychrotrophy.

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